EMBO reports

Manuscript EMBOR-2011-35188

Eomesodermin induces Mesp1 expression and cardiac differentiation from embryonic stem cells in the absence of Activin

Jelle van den Ameele, Luca Tiberi, Antoine Bondue, Catherine Paulissen, Adele Herpoel, Michelina Iacovino, Michael Kyba, Cedric Blanpain and Pierre Vanderhaeghen

Corresponding author: Pierre Vanderhaeghen, Universite Libre de Bruxelles ULB

Review timeline:

Submission date:	01 July 2011
Editorial Decision:	15 August 2011
Revision received:	15 December 2011
Editorial Decision:	11 January 2012
Revision received:	03 February 2012
Accepted:	06 February 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 August 2011

Thank you for the submission of your research manuscript to our editorial office. First of all, I would like to apologize for the unusual delay in getting back to you with a decision on your study, which was caused by the holiday season in which many referees need more time to complete their reports. We have now received the full sets of reviews on your study.

As the reports are pasted below I would prefer not to repeat them here in detail, but to only summarize the main points raised by the referees. You will see that, while the referees agree on the potential interest of the findings, they also feel that additional work is needed to strengthen the conclusions put forward.

Referee 2 and 3 state that the effects of Eomes on stem cell differentiation requires additional support. For example, both referees suggest expanding the analysis of marker gene expression for both Eomes- and activin-induced differentiation. Both reviewers also state that the effect of Eomes on Mesp1 expression should be tested in a transactivation assay; such an assay could also be useful in testing whether activin reduces binding of Eomes to the Mesp1 promoter (referee 3). Referee 1 feels that the effects of Eomes should be studied in vivo. However, we feel that this goes beyond the

scope of this report and would not be required if the in vitro effects are convincing. Having said this, I would suggest analyzing Eomes expression in vivo to test whether it is expressed at the right stage.

Overall, given the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees (as outlined above and in their reports) must be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

This manuscript is interesting to the community of CV developmental biologists, and further explores the regulation of MESP-1 and its role in cardiogenesis. MESP-1 plays a critical role in the early steps of mammalian cardiogenesis. The results identify eomesodermin as an upstream transcriptional regulator that binds directly to the MESP-1 promoter by ChIP analysis, and documents that inducible expression of eomesodermin can augment the expression of the downstream gene in an ES cell model of cardiogenesis. negative regulation of the effect by nodal/activin signaling is also noted and it is placed into a
framework that is consistent with our understanding of early steps of mesodermal specification.

At the same time, the study is largely based on in vitro studies and the exact role of eomesodermin is left to be fully resolved. As such, the impact of the study is more related to the finding of a an upstream regulator of MESP-1, an interesting finding but without a clear in vivo biological import to cardiogenesis. If this type of inn vivo data could be added it would considerably improve the manuscript. Also, it would be interest to see if the
heart progenitor intermediates which lie immediately downstream from MESP-1 lineages are preferentially affected in some way. This would broaden out the biological impact of the study and make it more relevant to a larger scientific community.

Referee #2:

van den Ameele et al show that in ES cells cultured in specific conditions favouring neural differentiation, overexpression of the T-box transcription factor Eomes promotes cardiac differentiation. It is not clear if this is an effect of promoting a specific cell type (in this case the authors focus on cardiac), or a general promotion of non-neural (specifically mesoderm) fates. Only select markers are chosen, and the numbers of cardiac cells is very small.

Quantitation for numbers of B-tubulin and Troponin T positive cells is required. This is shown only in Figure 3, and only for Troponin T. The numbers of B-tubulin + cells should be markedly reduced for the authors to conclude that differentiation had shifted towards a cardiac phenotype.

The reduction in Tubb3 mRNA levels by only 50% indicates that Eomes overexpression is not very potent in driving the differentiation of the ES cells away from a neural phenotype and towards a cardiac phenotype. Indeed, the highest induction of Troponin T+ cells, as shown in Fig 3B, is 5%, which is extremely low. Again, other markers should be used to quantitate the numbers of neural,

endoderm, and other mesodermal cell types.

The results with Activin are intriguing, but it is not explored how Activin switches the apparent specificity of Eomes. Without a mechanistic explanation, the results are perhaps interesting but without biological insight.

The link to Mesp1 activation is interesting. It would be useful to determine if the Eomes-bound region can respond in a transactivation assay, for example as part of a luciferase reporter construct. This would permit more extensive mechanistic investigation into the functionality of the binding site, and potential interacting proteins that could co-occupy adjacent regions in the presence or absence of activin.

Referee #3:

This manuscript addresses the role of Eomesodermin (Eomes) in mesendoderm differentiation by overexpression in embryonic stem cells. The main conclusion is that Eomes drives cardiogenesis, via binding and activiating Mesp, and that, in conjunction with Activin, Eomes promotes endoderm differentiation. While a high-level pulse of Eomes augments the formation of mesoderm and endoderm, further experiments are necessary to confirm the findings.

Only 5 % of the cell population express cardiac troponin (Fig. 3) despite almost DOX treated ESCs expressing Eomes (Supplementary Fig. 1). Therefore, the claim that Eomes drives cardiac mesoderm difficult to support because 95 % of the cells do not commit to the cardiac lineage. To establish that "with low levels of Activin, Eomes directly controls cardiac mesoderm specification" would require a more robust induction of cardiogenesis than that shown. It is more likely that the pulse of Eomes is permissive for mesoderm/endoderm formation in this media and a subset of these cells are capable of further differentiation along the cardiac lineage. Mesodermal induction should be established by examing the cultures by flow cytometry for early mesodermal markers such as PDGFRa and flk1 in the presence or absence of DOX. Similar studies have shown that a low percentage of ESCs overexpressing Eomes become PDGRRa/flk1 positive (Lindsley et al 2008). The authors should assess the extent of mesodermal differentiation of Eomes overexpressing cells in their defined culture conditions.

High levels of activin are known to drive endodermal differentiation. As for the mesoderm induction it would be useful to know the extent of endoderm induction using flow cytometry for markers such as CXCR4 and EpCAM. This would allow comparison of the differentiation outcomes due to Eomes overexpression. Overexpression of Eomes causes a moderate increase (2-3 fold) of Sox17 and slightly higher Gsc levels (4-5x) but the fold change is not significantly different between the two higher Activin levels (10 or 100 ng/ml). This increased expression of Sox17 and Gsc RNA in the DOX treated samples suggests that Eomes overexpression is permissive for further differentiation along the endodermal lineage. The lack of response between the two Activin levels is more puzzling as one might have expected a far greater number of endodermal cells to emerge under high Activin, especially as Eomes is proposed to "prime" the differentiating ESC to respond. However, it is difficult to draw strong conclusions from this data because the percentage of cells adopting endodermal cell fate is not shown. Quantification of endodermal cell fate would greatly strengthen the argument that in the presence of high activin Eomes drives endodermal differentiation.

The ChIP data are informative and demonstrate that Eomes is found at the promoters of Mesp1 and Mixl1 both in vivo and in vitro. Nevertheless, to validate this finding the authors should prove that Eomes is capable of directly transactivating the Mesp1 promoter in a heterologous assay system. Such data is necessary to confirm the hypothesis that Eomes activates Mesp1, and in this way is permissive for continued cardiogenesis.

Given the proposal that Eomes acts as an intrinsic switch controlling cell fate it would be interesting to determine if treatment with Activin reduced Eomes binding at the Mesp1 promoter. The displacement of Eomes from the Mesp1 promoter, but presumably not Mixl1 as it is expressed in both endodermal and mesodermal precursors in the primitive streak, would be expected under high levels of Activin. An alternative is that increased phospho-SMAD2/3 acts to block Mesp1

expression despite continued Eomes occupation of the promoter. This finding would be informative molecular steps behind the alternate cell fate choices.

Finally, the percentage of Sox17+ cells is not shown for the 100 ng/ml condition (Fig. 2 d), presumably it is the same as that seen for 10 ng/ml?

1st Revision - authors' response 15 December 2011

Referee #1:

This manuscript is interesting to the community of CV developmental biologists, ...it is placed into a framework that is consistent with our understanding of early steps of mesodermal specification. At the same time, the study is largely based on in vitro studies and the exact role of eomesodermin is left to be fully resolved. As such, the impact of the study is more related to the finding of a an upstream regulator of MESP-1, an interesting finding but without a clear in vivo biological import to cardiogenesis. If this type of in vivo data could be added it would considerably improve the manuscript.

We thank the Reviewer for his constructive and positive comments. We agree that in vivo data would indeed improve the manuscript, but we feel that this is not necessary for this manuscript to convey a convincing and high impact message. Indeed, while this work was in revision, the in vivo requirement of Eomes during cardiovascular specification has been nicely and convincingly illustrated by the work of Costello and colleagues, which clearly demonstrated through the use of chimeric embryos that Eomes deficient cells could not contribute to the cardiac lineages and that Eomes controls Mesp1 induction in the mouse embryo (Costello et al., 2011).
On the other hand our manuscript, thanks to the use of a serum free medium supplemented with defined morphogens, not only reveals an intrinsic pathway of cardiac commutation triggered by Eomes and Mesp1, but also its elaborate relationships with extrinsic cues such as Activin. Our work thus clearly represents a significant advance of our understanding of the mechanisms that regulate cardiovascular cell fate specification despite the fact that it is largely based on in vitro data, with the notable exception of in vivo ChiP experiments that constitute the first evidence for a direct interaction between Eomes and Mesp1 in vivo.

Also, it would be interest to see if the heart progenitor intermediates which lie immediately downstream from MESP-1 lineages are preferentially affected in some way. This would broaden out the biological impact of the study and make it more relevant to a larger scientific community.

We agree with the reviewer's comment that the demonstration that Eomes promotes the specification of cardiovascular progenitors that lie immediately downstream of Mesp1 would broaden the biological impact and make it more relevant for the scientific community. We have now addressed this important point by quantifying the proportion of the cardiovascular progenitors that lie immediately downstream of Mesp1 using Pdgfra/Flk1 FACS analyses (Bondue et al Journal of Cell Biology 2011 and Kattman et al. Cell Stem Cell 2011) (Figure 3). These analyses indicate that Eomes induction is followed by a four-fold increase in the proportion of Pdgfra/Flk1 -expressing heart progenitors 48 hours after induction of Eomes (from 1.6 to 7.4%), consistent with the promotion of multipotent cardiovascular progenitors that lie downstream of Mesp1 by Eomes.

Referee #2:

van den Ameele et al show that in ES cells cultured in specific conditions favouring neural differentiation, overexpression of the T-box transcription factor Eomes promotes cardiac differentiation. It is not clear if this is an effect of promoting a specific cell type (in this case the authors focus on cardiac), or a general promotion of non-neural (specifically mesoderm) fates. Only select markers are chosen, and the numbers of cardiac cells is very small.

We thank the Reviewer for his constructive and positive comments. We have now extended considerably our marker analyses, both qualitatively and quantitatively, using FACS, immunostainings and qRTPCR (Figures 1,2,3). These new results show that the three cardiovascular cell lineages (cardiac, endothelial and smooth muscle cells) represent more than 80% of the cells after 10 days of ESC differentiation following Eomes overexpression in the absence of activin, as explained in further details below.

Quantitation for numbers of B-tubulin and Troponin T positive cells is required. This is shown only in Figure 3, and only for Troponin T. The numbers of B-tubulin + cells should be markedly reduced for the authors to conclude that differentiation had shifted towards a cardiac phenotype.
The reduction in Tubb3 mRNA levels by only 50% indicates that Eomes overexpression is not very potent in driving the differentiation of the ES cells away from a neural phenotype and towards a cardiac phenotype.
Indeed, the highest induction of Troponin T+ cells, as shown in Fig 3B, is 5%, which is extremely low. Again, other markers should be used to quantitate the numbers of neural, endoderm, and other mesodermal cell types.

We have now quantified more thoroughly markers of neural and mesodermal/cardiovascular fate expression, using immunostainings on cytospin preparations and FACS.
As for neural fate, our new data indicate a drastic reduction (from 8,2% to 0,5%) in the proportion of tubb3+ cells, thus corresponding to neurons, following Eomes induction in absence of activin (Figure 1); this is a much more reliable indication of inhibition of neuronal fate than qrtpcr for tubb3, which in our experience is a poor indicator of the actual number of neuronal cells (Gaspard et al. Nature Protocols 2009 and our unpublished data). In addition Sox1, a generic marker of neural progenitors, is also drastically reduced following Eomes induction, providing further evidence of inhibition of neural fate acquisition by Eomes.
We have also further examined markers of derivatives of cardiovascular progenitors, including Troponin, CD31 and Smooth muscle actin (SMA). As for troponin, additional FACS analyses revealed that up to 25% of Tnnt2-positive cells can be obtained following Eomes induction (Figure 1). This is even higher than the range (5-10%) described in the first version of the manuscript. This improvement in spontaneous and Eomes-induced cardiac differentiation could be due to the use of different lots of differentiation components (such as N2) and better optimization of ES cell culture before the differentiation. In addition and importantly, the proportion of cells expressing either CD31 or SMA displays a major increase following Eomes induction (from 7,0 to 54,5% and from 8,7 to 64,5%, respectively).
Finally analysis of Sox17 expression indicates rates of less than 10% of cells expressing this endodermal marker, confirming the marginal endodermal fate acquisition in the absence of activin.
Overall these data confirm our previous analyses, and indicate that, following induction of Eomes in the absence of activin, the vast majority of the cells have acquired a cardiovascular fate.

The results with Activin are intriguing, but it is not explored how Activin switches the apparent specificity of Eomes. Without a mechanistic explanation, the results are perhaps interesting but without biological insight.

The role of Activin in promoting endoderm specification during ESC differentiation, as well as the role of Eomes in promoting endoderm specification from ESC in the presence of activin is well established (Kubo et al. Development 2004, Gadue et al. PNAS 2006, D'Amour et al. Nat. Biot. 2005, Yasunaga et al. Nat. Biot. 2005, Borowiak et al. Cell Stem Cell 2009, Teo et al. Genes & Dev 2011). The novelty of our finding is not that Eomes promotes endoderm specification in the presence of activin, but rather that Eomes promotes cardiac specification in the absence of activin. Our results suggest that in the absence of activin Eomes promotes Mesp1 expression, which in turn promotes cardiac differentiation, and represses endoderm key transcription factors such as Sox17 or Foxa2 (Bondue et al. Cell Stem Cell 2008), explaining the specific cardiovascular fate promotion induced by Eomes in this assay. We believe that our demonstration that Eomes-dependent cell fate acquisition is switched in the presence of activin, also provides direct and important biological insights into cardiovascular progenitor specification. In addition our new additional ChiP analyses of Eomes binding to the Mesp1 promoter in the presence or absence of activin revealed a decrease in the recruitment of Eomes to the Mesp1 promoter in the presence of activin (Figure 4). These data suggest that binding of Eomes to the Mesp1 promoter could be differentially affected by activin, which could explain why Mesp1 is not expressed in endodermal cells expressing Eomes, and provide at least in part a molecular mechanism underlying Eomes/activin-dependent fate switch.

The link to Mesp1 activation is interesting. It would be useful to determine if the Eomes-bound region can respond in a transactivation assay, for example as part of a luciferase reporter construct. This would permit more extensive mechanistic investigation into the functionality of the binding site, and potential interacting proteins that could co-occupy adjacent regions in the presence or absence of activin.

We have performed extensive heterologous assays to test direct transactivation of the Mesp1 promoter by Eomes. We used Mesp1 promoter constructs previously validated in vivo and in ES cells (Haraguchi et al. Mech. Dev. 2001, Oginuma et al. Mech. Dev. 2008, Bondue et al. JCB 2011 and our unpublished data), containing up to 5.6kb of the proximal regulatory regions of the mouse Mesp1 gene, driving luciferase or GFP reporters.
We first tried to transiently transfect ESC with our reporter constructs using electroporation and nucleofection. However the low level of transfection (the length of plasmids containing the reporter constructs is greater than 10KB) and the poor survival after transfection did not allow to perform these reporter assays in ESC in a reliable manner.
We next tried to perform these assays in several cell lines (Hek293T, Cos7, CHO), including P19 cells which are capable of cardiac differentiation upon DMSO administration using a promoter previously validated during ESC differentiation in vitro (Bondue et al JCB 2011) and during mouse development vivo (Haraguchi et al. Mech. Dev. 2001 and our unpublished data). Somewhat surprisingly, we were not able to detect significant transactivation in any condition tested. These negative results could reflect several technical issues, such as weak promoter activity or inappropriate cellular context for proper transactivation (such as the absence of a particular factor that would be required with a precise stoichiometry for Eomes to work properly on the Mesp1 promoter), or the need for additional epigenetic factors that would not function properly in transient transfection assays.
Nevertheless, even in absence of transactivation data, our data strongly suggest that Eomes acts at least in part through Mesp1 to achieve cardiac induction. This is further supported by additional experiments, where we have tested the requirement of Mesp1 activity in the effect of Eomes on cardiovascular fate induction (Figure 4). Specifically, we found that the cardiac fate induced by Eomes is strongly inhibited by co-expression of a Mesp1-Engrailed fusion factor, which acts as a Mesp1 dominant-negative inhibitor (Bondue et al., 2011). While these data further link Mesp1 and Eomes upstream of cardiac induction, they also indicate that Eomes is likely to act both directly and indirectly on Mesp1 expression, and that additional Eomes target genes are involved in the specification of cardiovascular fate in the absence of growth factors, as discussed in the revised version of the manuscript.

Referee #3:

...While a high-level pulse of Eomes augments the formation of mesoderm and endoderm, further experiments are necessary to confirm the findings. Only 5 % of the cell population express cardiac troponin (Fig. 3) despite almost DOX treated ESCs expressing Eomes (Supplementary Fig. 1). Therefore, the claim that Eomes drives cardiac mesoderm difficult to support because 95 % of the cells do not commit to the cardiac lineage. To establish that "with low levels of Activin, Eomes directly controls cardiac mesoderm specification" would require a more robust induction of cardiogenesis than that shown. It is more likely that the pulse of Eomes is permissive for mesoderm/endoderm formation in this media and a subset of these cells are capable of further differentiation along the cardiac lineage. Mesodermal induction should be established by examining the cultures by flow cytometry for early mesodermal markers such as PDGFRa and flk1 in the presence or absence of DOX. Similar studies have shown that a low percentage of ESCs overexpressing Eomes become PDGRRa/flk1 positive (Lindsley et al 2008). The authors should assess the exte nt of mesodermal differentiation of Eomes overexpressing cells in their defined culture conditions.

We thank the Reviewer for his constructive and positive comments.
We have further examined markers of cardiovascular fate, including Troponin, CD31 and Smooth muscle actin (SMA). As for troponin, additional FACS analyses revealed that up to 25% of Tnnt2-positive cells can be obtained following Eomes induction. This is even higher than the range (5-10%) described in the first version of the manuscript. This improvement in cardiac induction could be due to the use of different lots of differentiation components (such as N2) and better optimization of ES cell culture before the differentiation. In addition and importantly, the proportion of cells expressing either CD31 or SMA display a major increase following Eomes induction (from 7,0 to 54,5% and from 8,7 to 64,5%, respectively). Analysis of Sox17 expression indicates rates of less than 10% of cells expressing this endodermal marker, confirming marginal endodermal fate acquisition in the absence of activin.
Overall these data confirm our previous analyses, and indicate that, following induction of Eomes in the absence of activin, the vast majority of the cells have acquired a cardiovascular fate.
On the other hand, even though cardiovascular induction is robust and suggestive of instructive fate changes, we do agree with the Reviewer that our data do not allow to rule out an additional permissive role for Eomes in the acquisition of mesodermal and endodermal fates, which is now discussed in the revised version of the manuscript.

High levels of activin are known to drive endodermal differentiation. As for the mesoderm induction it would be useful to know the extent of endoderm induction using flow cytometry for markers such as CXCR4 and EpCAM. This would allow comparison of the differentiation outcomes due to Eomes overexpression. Overexpression of Eomes causes a moderate increase (2-3 fold) of Sox17 and slightly higher Gsc levels (4-5x) but the fold change is not significantly different between the two higher Activin levels (10 or 100 ng/ml). This increased expression of Sox17 and Gsc RNA in the DOX treated samples suggests that Eomes overexpression is permissive for further differentiation along the endodermal lineage. The lack of response between the two Activin levels is more puzzling as one might have expected a far greater number of endodermal cells to emerge under high Activin, especially as Eomes is proposed to "prime" the differentiating ESC to respond. However, it is difficult to draw strong conclusions from this data because the percentage of cells adopting endodermal cell fate is not shown. Quantification of endodermal cell fate would greatly

strengthen the argument that in the presence of high activin Eomes drives endodermal differentiation.

We have now quantified as suggested by the reviewer the extent of endodermal differentiation following Eomes in absence and presence of activin at early time points using CXCR4/EpCAM and Sox17 markers (Figure 3). This analysis revealed that up to 40-50% of the cells express endodermal fate markers in the presence of 10 or 100 ng/ml of activin (Figure 3). These data indicate that endodermal fate is indeed induced by Eomes when activin is present, in good accordance with results previously published by other groups (Izumi et al. Stem Cells 2007, Teo et al. G&D. 2011).

The ChIP data are informative and demonstrate that Eomes is found at the promoters of Mesp1 and Mixl1 both in vivo and in vitro. Nevertheless, to validate this finding the authors should prove that Eomes is capable of directly transactivating the Mesp1 promoter in a heterologous assay system. Such data is necessary to confirm the hypothesis that Eomes activates Mesp1, and in this way is permissive for continued cardiogenesis.

We have performed extensive heterologous assays to test direct transactivation of the Mesp1 promoter by Eomes. We used Mesp1 promoter constructs previously validated in vivo and in ES cells (Haraguchi et al. Mech. Dev. 2001, Oginuma et al. Mech. Dev. 2008, Bondue et al. JCB 2011 and our unpublished data) , containing up to 5.6kb of the proximal regulatory regions of the mouse Mesp1 gene, driving luciferase or GFP reporters.
We first tried to transiently transfect ESCs with our reporter constructs using electroporation and nucleofection. However the low level of transfection (the length of plasmids containing the reporter constructs is greater than 10KB) and the poor survival after transfection did not allow to perform these reporter assays in ESC in a reliable manner.
We next tried to perform these assays in several cell lines (Hek293T, Cos7, CHO), including P19 cells which are capable of cardiac differentiation upon DMSO administration using a promoter previously validated in ESC in vitro (Bondue et al JCB 2011) and during mouse development vivo (Haraguchi et al. Mech. Dev. 2001 and our unpublished data). Somewhat surprisingly, we were not able to detect significant transactivation in any condition tested. These negative results could reflect several technical issues, such as weak promoter activity or inappropriate cellular context for proper transactivation (such as the absence of a particular factor that would be required with a precise stoichiometry for Eomes to work properly on the Mesp1 promoter), or the need for additional epigenetic factors that would not function properly in transient transfection assays.
Nevertheless, even in absence of transactivation data, our data strongly suggest that Eomes acts at least in part through Mesp1 to achieve cardiac induction. This is further supported by additional experiments, where we have tested the requirement of Mesp1 activity in the effect of Eomes on cardiovascular fate induction (Figure 4). Specifically, we found that the cardiac fate induced by Eomes is strongly inhibited by co-expression of a Mesp1-Engrailed fusion factor, which acts as a Mesp1 dominant-negative inhibitor (Bondue et al., 2011). While these data further link Mesp1 and Eomes upstream of cardiac induction, they also indicate that Eomes is likely to act both directly and indirectly on Mesp1 expression, and that additional Eomes target genes are involved in induction of cardiovascular fate, as discussed in the revised version of the manuscript.

Given the proposal that Eomes acts as an intrinsic switch controlling cell fate it would be interesting to determine if treatment with Activin reduced Eomes binding at the Mesp1 promoter. The displacement of Eomes from the Mesp1 promoter, but presumably not Mixl1 as it is expressed in both endodermal and mesodermal precursors in the primitive streak, would be expected under high levels of Activin. An alternative is that increased phospho-SMAD2/3 acts to block Mesp1 expression despite continued Eomes occupation of the promoter. This finding would be informative molecular steps behind the alternate cell fate choices.

We performed additional ChiP analyses of Eomes binding to the Mesp1 promoter in the presence or absence of activin. Interestingly, this revealed decreased recruitment of Eomes to the Mesp1

promoter in the presence of activin (Figure 4). These data suggest that the binding of Eomes to the Mesp1 promoter could be differentially affected by activin, although they could also reflect activin-dependent changes in cell fate.

Finally, the percentage of Sox17+ cells is not shown for the 100 ng/ml condition (Fig. 2 d), presumably it is the same as that seen for 10 ng/ml?

The number of Sox17-positive cells is now provided for all conditions (figure 3).

2nd Editorial Decision 11 January 2012

Many thanks for submitting your revised manuscript to our editorial office. I would like to apologize for the delay in the review process of your study, which was due to the recent holiday season. The manuscript was sent to two of the original reviewers, and we have now received their reports on it.

As you will see, while referee #3 now supports publication, referee #2 is still concerned about the efficiency of cardiac differentiation and the lack of a response in the in vitro transactivation assay. With regard to the latter point, referee 3 suggests to show and discuss this result and I agree with him/her that this would be sufficient. With regard to the efficiency of Eomes-mediated cardiac differentiation and to strengthen the statistical significance of this result, I would ask you to show all individual data points from the three independent experiments in one plot. The remaining concerns brought up by both referees should also be addressed, but they seem to be rather minor. In additional communications between referee 3 and the editorial office, this referee pointed out that it should be made clear that it is likely that not all cells go through a Mesp1 stage. For example, s/he states that the CD31-positive endothelial cells could have arisen from hemangioblasts that migrate through the primitive streak at a similar time to cardiac progenitors and this should be stated more clearly.

I would kindly ask you to address these remaining points before acceptance of the manuscript. Once you have done so, please upload the final version through our website again. Formally, this needs to be done before February 15th, as the paper needs to be accepted at the latest six months after the initial decision, which in your case was made on August 15th.

I should also point out that we have recently changed our reference style to a number-based on. Therefore, may I ask you to change the reference style according to the new format? The details of this and the necessary end note file can be found here:

http://www.nature.com/embor/about/authors.html#refformat

I am truly sorry for this inconvenience and hope this will not turn out to be too difficult.

I thank you in advance for your cooperation. Please do not hesitate to contact me if you have any further questions.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #2:

van den Ameele have revised their manuscript and provided additional data in support of their contention that Eomes directly activates Mesp1 to promote cardiac differentiation. Despite these improvements, some important points are not suitably addressed, and the recent publication

demonstrating the in vivo regulation of Mesp1 by Eomes is supportive but takes away from novel aspects of the current study.

An important point was the low efficiency of cardiac differentiation in the context of Eomes activation. While the authors now report much higher % cTnT+ cells, it is unclear what the basis for this is. The authors' speculation about media lots and improvements to the protocol do not instill confidence that these results are reproducible, and certainly do not provide a direct link to cardiovascular cell type differentiation.

The basis for the activin-mediated switch is still unexplained, and thus remains phenomenological.

The lack of direct transactivation of the Mesp1 regulatory elements by Eomes is still quite problematic, and indicates that the regulation is likely to be indirect.

In the in vivo ChIP, it is not clear what the Mesp1 (-) condition is; how can there be a condition without Mesp1, unless these are Mesp1-null embryos.

Referee #3:

The re-submitted manuscript has sufficient new data to address previous concerns. Further, the results from this manuscript provide support to those published during the review period. The following minor points should be addressed before publication:

i) the results from the experiments to transactivate the Mesp1 promoter with Eomes should be included and mentioned. Although "negative" the requirement for the appropriate co-factors is of interest to the field. The data can be included in supplementary figure 3.
ii) representative FACS plots for PDGF/Flk1 and Epcam/Cxcr4 should be presented, these can be included in supplemental data.

2nd Revision - authors' response 03 February 2012

Referee #2:

van den Ameele have revised their manuscript and provided additional data in support of their contention that Eomes directly activates Mesp1 to promote cardiac differentiation. Despite these improvements, some important points are not suitably addressed,... An important point was the low efficiency of cardiac differentiation in the context of Eomes activation. While the authors now report much higher % cTnT+ cells, it is unclear what the basis for this is. The authors' speculation about media lots and improvements to the protocol do not instill confidence that these results are reproducible, and certainly do not provide a direct link to cardiovascular cell type differentiation.

There is indeed some inherent variability in the precise percentage obtained for cTnT-positive cells obtained in different experiments. The reason for this remains unclear, and in our experience is highly likely to be linked to changes in the quality of lots of culture reagents, which is a well known issue in ESC culture and differentiation. To account for this useful information, we now provide the scatter plots representing the complete raw data sets for all cTnT FACS experiments that we performed for this study (Supplementary figure 4, relating to figure 1I, figure 2A and figure 4D). While we hope that these full data sets will be useful to the field, we strongly believe that our data still indicate a strong promoting effect of Eomes on cardiovascular differentiation from ESC in the absence of activin, even considering the few data obtained in suboptimal conditions.

The basis for the activin-mediated switch is still unexplained, and thus remains phenomenological.

As explained before, our demonstration that Eomes can drive differential fates depending on extrinsic activin is a novel and mechanistically relevant finding. In addition our new data from ChIP analyses of the Mesp1 promoter in the presence or absence of activin strengthen the notion that the responsiveness to Eomes can be switched depending on the context in which the cells develop. Our study thus paves the way to dissect further the precise molecular mechanisms underlying this molecular switch, with important implications for our understanding of early germ layer specification and directed differentiation of ESC.

The lack of direct transactivation of the Mesp1 regulatory elements by Eomes is still quite problematic, and indicates that the regulation is likely to be indirect.

As explained before, we have performed extensive heterologous assays to test direct transactivation of the Mesp1 promoter by Eomes. We used Mesp1 promoter constructs previously validated in vivo and in ES cells (Haraguchi et al. Mech. Dev. 2001, Oginuma et al. Mech. Dev. 2008, Bondue et al. JCB 2011 and our unpublished data).
We first tried to transiently transfect ESC with our reporter constructs but the low level of transfection and the poor survival after transfection did not allow to perform these assays in a reliable manner. We next tried several cell lines (Hek293T, Cos7, CHO), including P19 cells that are capable of cardiac differentiation upon DMSO administration. Somewhat surprisingly, we were not able to detect significant transactivation in any condition tested. These negative results could reflect several technical issues, such as weak promoter activity or inappropriate cellular context for proper transactivation (such as the absence of a particular factor that would be required with a precise stoichiometry for Eomes to work properly on the Mesp1 promoter), or the need for additional epigenetic factors that would not function properly in transient transfection assays. We have now included some of these experiments in Supplementary figure 3.
Nevertheless, even in the absence of transactivation results, our data strongly suggest that Eomes acts at least in part through Mesp1 to achieve cardiac induction. This is further supported by additional experiments, where we found that the cardiac fate induced by Eomes is strongly inhibited by co-expression of a Mesp1 dominant-negative inhibitor. While these data further link Mesp1 and Eomes upstream of cardiac induction, they also indicate that Eomes is likely to act both directly and indirectly on Mesp1 expression, and that additional Eomes target genes are involved in the specification of cardiovascular fate in the absence of growth factors, as discussed in the revised version of the manuscript.

In the in vivo ChIP, it is not clear what the Mesp1 (-) condition is; how can there be a condition without Mesp1, unless these are Mesp1-null embryos.

The Mesp1 (-) refers to the negative control site depicted in Figure 4. This is now clarified in the corresponding legend.

Referee #3:

The re-submitted manuscript has sufficient new data to address previous concerns. Further, the results from this manuscript provide support to those published during the review period. The following minor points should be addressed before publication:

i) the results from the experiments to transactivate the Mesp1 promoter with Eomes should be included and mentioned. Although "negative" the requirement for the appropriate co-factors is of interest to the field. The data can be included in supplementary figure 3.

We have now included some of the experiments performed with Mesp1 promoter reporters in P19 cells in Supplementary figure 3.

ii) representative FACS plots for PDGF/Flk1 and Epcam/Cxcr4 should be presented, these can be included in supplemental data.

Representative density plots are now included in supplementary figure 2E-F.

3rd Editorial Decision 06 February 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

